Expression and Polarized Targeting of a Rab3 Isoform in Epithelial Cells

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Abstract. Pathways of polarized membrane traffic in epithelial tissues serve a variety of functions, including the generation of epithelial polarity and the regulation of vectorial transport. We have identified a candidate regulator of polarized membrane traffic in epithelial cells (i.e., rab3B), which is a member of the rab family of membrane traffic regulators. Rab3B is highly homologous to a brain-specific rab3 isoform (rab3A) that targets in a polarized fashion to the presynaptic nerve terminal, where it probably regulates exocytosis. The coding region for human rab3B was cloned from epithelial mRNA using a reverse-transcription polymerase chain reaction strategy. This cDNA clone hybridized to a single mRNA species in Northern blots of poly(A)$^+$ RNA isolated from epithelial cell lines. A rab3B-specific antibody that was raised against recombinant fusion protein recognized a 25-kD band in immunoblots of cell lysates prepared from cultured epithelial cells (e.g., T_{84} and HT29-CL19A), but not from a variety of nonepithelial cells (e.g., PC12 neuroendocrine cells). Immunofluorescence analysis confirmed that rab3B protein is preferentially expressed in cultured epithelial cells as well as in a number of native epithelial tissues, including liver, small intestine, colon, and distal nephron. Rab3B localized to the apical pole very near the tight junctions between adjacent epithelial cells within all of these cell lines and native epithelial tissues, as determined by immunofluorescence and immunoelectron microscopic analysis. Moreover, this pattern of intracellular targeting was regulated by cell contact; namely, rab3B was reversibly retrieved from the cell periphery as epithelial cell contact was inhibited by reducing the extracellular Ca^{2+} concentration. Our results indicate that neurons and epithelial cells express homologous rab3 isoforms that target in a polarized fashion within their respective tissues. The pattern and regulation of rab3B targeting in epithelial cells implicates this monomeric GTPase as a candidate regulator of apical and/or junctional protein traffic in epithelial tissues.

EPITHELIAL tissues engage in two major functions: (*a*) the vectorial transport of macromolecules, small solutes, or water, and (*b*) the maintenance of transepithelial solute gradients by serving as selective barriers to diffusion. The capacity for vectorial transport absolutely depends on the ability of individual epithelial cells to generate and maintain a polarized phenotype; i.e., to polarize into distinct apical and basolateral membrane domains with different transport properties. The barrier function depends in turn on the existence of tight junctions, which serve as perm-selective gates to paracellular solute flow and as fences to minimize the lateral mixing of apical and basolateral molecules. The development of the polarized epithelial phenotype and the biogenesis of tight junctions involve polarized pathways of vesicle trafficking that selectively target proteins and lipids to either plasma membrane domain, as well as to the junctional complexes (22, 23). Such pathways of polarized targeting also participate in acutely regulating the functional properties of fully differentiated epithelial cells; e.g., by mediating protein secretion across the apical or basolateral membranes (27, 35), or the regulated insertion of transport proteins into either plasma membrane domain (e.g., apical water channels in vasopressin-stimulated epithelia [3]). Clearly, such pathways of polarized membrane traffic are important elements in the process of epithelial cell differentiation and the regulation of epithelial cell function.

The intracellular traffic of apical and basolateral proteins is subject to multiple levels of regulation, including initial sorting at the TGN and selective targeting between the TGN and the relevant plasma membrane domain (22, 23). Such regulation likely involves the participation of a series of molecules that control polarized membrane traffic, some of which may reside exclusively within the TGN and others of which may function downstream of the TGN at or near each

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membrane domain (22, 23). On the basis of these considerations, investigators have searched for downstream regulators of vesicle traffic that target in a polarized fashion within epithelial cells (6). This search has focused in large part on members of the rab family of monomeric GTPases (5, 39); proteins that are homologous to several regulators of secretion by yeast (i.e., SEC4 and YPT1; [11, 32]). Rabs target to distinct membrane compartments (e.g., rab 5 to plasma membrane and endosomes; [5]), where they regulate specific pathways of membrane traffic (e.g., endocytosis, in the case of rab5; [4]). Although most rabs appear to be expressed in a wide variety of tissues, one member of this family has been shown to be preferentially expressed in epithelial cells (i.e., rab 17; [17]). Interestingly, rab 17 is not expressed in a polarized fashion within epithelial cells (i.e., it targets to vesicles in both the apical and basal poles of epithelial cells), which has led Lütcke et al. (17) to speculate that this protein regulates transcytosis across epithelial tissues.

In the present study we have identified a candidate regulator of membrane traffic that targets in a polarized fashion within epithelial cells; namely, rab3B. This protein is of particular interest because it is highly homologous (78% amino acid identity) to a rab3 isoform (rab3A) that is preferentially expressed in neurons, where it specifically associates with synaptic vesicles and appears to mediate neurotransmitter release (9, 19, 20, 24, 26, 33). The cDNA encoding rab3B was originally cloned from brain; however, unlike rab3A, rab3B is expressed at very low levels in brain (14, 19). We show by several criteria that rab3B is preferentially expressed in a variety of cultured epithelial cell lines and in native epithelial tissues (i.e., liver, intestine, and kidney). Like rab3A in neurons, this protein targets in a polarized fashion within epithelial cells, namely, to the apical pole in close proximity to epithelial tight junctions. In addition, this pattern of intracellular targeting is regulated by cell contact; i.e., rab3B is reversibly retrieved from the cell periphery as epithelial cells are dissociated by reducing the extracellular calcium concentration. Our results indicate that epithelial cells and neurons express homologous rab3 isoforms which exhibit polarized targeting in their respective tissues, and implicate rab3B as a regulator of the traffic of apical and/or junctional proteins in epithelial cells.

Materials and Methods

Cell Culture

All cells cultured for analysis of RNA or protein were grown in plastic tissue culture flasks. Epithelial cells used for immunofluorescence were cultured on glass coverslips or on transparent permeable filters (Cyclopore [Falcon Plastics, Cockeysville, MD]; 0.45-μm pore diameter), the latter of which were used to optimize the development of cell polarity. Cells were maintained at 37°C in 5% CO_2 in the following media: DME + 10% FBS (L cells and HT-29 CL-19A), DME-F12 + 10% FBS (T_{84}), IMDM + 10% FBS (CFPAC), RPMI + 10% FBS and 5% horse serum (PC12), McCoy's 5A + 10% FBS (parental HT29), and M199 + 10% FBS (bovine aortic endothelial cells).

Reverse Transcription/Polymerase Chain Reaction (RT-PCR)

Poly(A)$^+$ RNA was isolated from cells cultured in T_{75} flasks (75–100% confluency) using the Fastrack Kit from Invitrogen (San Diego, CA). Each RNA pellet was resuspended in 20 μl H_2O and 3 μl was reverse transcribed into cDNA using random hexamers (RT-PCR kit; Perkin Elmer, Norwalk, CT). The cDNA was then amplified by 30 cycles of PCR using amplimers specific for the 5′ and 3′ ends of the published coding regions of human rab3B and rab 5 (39). The upstream and downstream amplimers contained adapters encoding unique BamHI and EcoRI cleavage sites, respectively, in order to facilitate directional cloning into the prokaryotic expression vector, pGEX-2T (see below). The amplimer sequences, with enzyme sites in parentheses, were as follows:

Rab3B (upstream):
5′-GATC(GGATCC)ATGGCTTCAGTGACAGATGGTAAA-3′
Rab3B (downstream):
5′-GATC(GAATTC)TTGCTAGCATGAGCAGTTCTGCTG-3′
Rab 5 (upstream):
5′-GATC(GGATCC)ATGGCTAGTCGAGGCGCAACAAGA-3′
Rab 5 (downstream):
5′-CGGC(GAATTC)GGTTTAGTTACTACAACACTG-3′

The resulting PCR products were subcloned into pGEX-2T and sequenced using standard dideoxy sequencing techniques.

Northern Blots

Poly(A)$^+$ RNA isolated as described above was resolved on formaldehyde/agarose gels (1%) and then stained with ethidium bromide (1 μg/ml for 45 min) to visualize the 28S and 18S ribosomal RNA bands (i.e., to confirm RNA integrity). Each gel was then washed free of ethidium bromide and the RNA transferred to nylon membrane (Hybond; Amersham Corp., Arlington Heights, IL) by capillary blotting. Each blot was hybridized with the full-length clone of the rab3B-coding region (see above) labeled with [α-^{32}P]dCTP by the random primer labeling method (Prime-a-Gene labeling system; Promega Corp., Madison, WI). Hybridization and subsequent washes were performed under conditions of high stringency; i.e., hybridization at 65°C for 12 h followed by consecutive washes with 2× SSC (150 mM NaCl, 15 mM NaCitrate, pH 7.0), 0.1% SDS (22°C), 0.5× SSC, 0.1% SDS (22°C), and 0.1× SSC, 0.1% SDS (65°C). Autoradiography was performed for 24–48 h at −70°C with intensifying screens.

Generation and Purification of Polyclonal Antibodies Against Recombinant Rab3B

The coding region for rab3B was cloned into the BamHI and EcoRI sites of the pGEX-2T vector located downstream from the coding sequence for glutathione-S-transferase (GST)[1]. Transformed *Escherichia coli* were induced with 0.1 mM IPTG to produce GST-rab3B fusion protein that was then affinity purified from bacterial cell lysates with glutathione-agarose, as described (34). Fusion protein was eluted from glutathione-agarose in 8 M urea and dialyzed overnight against TBS (50 mM Tris [pH 7.5], 150 mM NaCl). GST-rab3B fusion protein was injected into rabbits for antibody production and IgG was purified from antisera as described (13). Antibodies specific for the hypervariable COOH-terminal domain of rab3B were generated by affinity purifying the IgG fraction against GST-Crab3B, where CRab3B represents a COOH-terminal, polypeptide fragment of rab3B (amino acids 168–219). The cDNA encoding CRab3B was generated from the full-length rab3B clone by PCR using the downstream rab3B amplimer (see above) and the following upstream primer: 5′-GATC(GGATCC)-GAGAACATCAGTGTAAGGCAGGCC-3′. The resulting PCR product was cloned into pGEX-2T and translated as a fusion with GST (as described above). GST-CRab3B was then affinity purified, covalently coupled to tresyl-activated agarose beads (Schleicher & Schuell, Inc., Keene, NH), and used to affinity purify the IgG from antisera. The affinity-purified antibody was then cleared of GST antibodies by absorption against excess GST coupled to agarose.

We also produced GST-rab3A and GST-rab5 fusion proteins for the purpose of characterizing antibody specificity. The coding region for rab5 was generated by RT-PCR as described for rab3B. The cDNA encoding bovine rab3A was kindly provided by T. Südhof (University of Texas/Southwestern, Dallas, TX). Each of these cDNAs was ligated into pGEX-2T and expressed in bacteria as the appropriate GST fusion protein.

Immunoblots

Immunoblots of fusion proteins and membrane proteins prepared as de-

1. *Abbreviations used in this paper*: ECL, enhanced chemiluminescence; GST, glutathione-S-transferase.

scribed below were performed as follows. Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose. Nitrocellulose membranes were washed with TBS, blocked with 5% (wt/vol) dry milk in TBS, and incubated overnight at 4°C with either preimmune IgG, immune IgG, or affinity-purified antibody in TBS plus 1.0 μg/ml leupeptin. Blots were reblocked with dry milk and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2,500 dilution) for 1 h at room temperature. Blots were developed using the enhanced chemiluminescence (ECL) kit from Amersham Corp.

Immunoblots of cell lysates were performed as follows. Cells were washed in PBS and then lysed for 40 min at 4°C in lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Hepes [pH 7.0], 1 mM EDTA, 1% aprotinin, and 1 mM PMSF). Lysates were centrifuged at 16,000 *g* for 15 min to pellet nuclei and insoluble material. Protein concentrations of cell lysates were determined using the micro BCA protein assay kit from Pierce Chem. Co. (Rockford, IL). Lysed proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Bio Rad Labs., Hercules, CA), as described (29). Membranes were rinsed in TBS, blocked in 5% dry milk plus 0.1% Tween 20 in TBS, and then incubated with the primary antibody for 1 h at room temperature in 1 M glucose, 0.5% Tween 20, 10% glycerol, and 5% dry milk in TBS. Each membrane was then washed in 0.1% Tween 20, reblocked in 5% dry milk plus 0.1% Tween 20, and then exposed to the secondary antibody (HRP-conjugated anti-rabbit IgG; 1:2,500 dilution) as described for the primary antibody. The membrane was then washed extensively in 0.1% Tween 20 and developed by ECL.

Membrane Preparations

The indicated cultured cells were homogenized in sucrose buffer (6.5–7.5%) and centrifuged at 3,000 *g* for 10 min to pellet nuclei and unbroken cells. The supernatant was then centrifuged at 100,000 *g* for 1 h to provide a crude membrane preparation (pellet) and cytosol (supernatant). Light microsomes were prepared from the crude membrane pellet by the method of Reinhardt et al. (28). Briefly, the pellet was resuspended in homogenization buffer and applied to a discontinuous sucrose density gradient. The gradient was centrifuged at 110,000 *g* for 90 min and the 20/40% (wt/vol) sucrose interface collected. This interface represents a light microsome fraction enriched in endosomes and plasma membrane markers relative to markers of organelles (e.g., mitochondria and endoplasmic reticulum; [28]). Protein concentrations were determined by the Bradford assay using bovine serum albumin standards.

Expression of Recombinant Rab3B and Rab3A in COS-7 Cells

The coding region of rab3B was ligated into the BamHI–EcoRI cloning site of the mammalian expression vector, pCDNA-1 (Invitrogen). COS-7 cells were transfected by electroporation with this construct or a corresponding rab3A construct (pCMVb-25-1a; provided by T. Südhof), as follows. First, cells grown to 100% confluency in T_{75} flasks were suspended in phosphate-buffered sucrose (272 mM sucrose; pH 7.35). Plasmid DNA was added to a final concentration of 20–40 μg/ml. The cells were then electroporated (Gene Pulse; Bio Rad Laboratories, Cambridge, MA), seeded onto 35-mm dishes in DME + 10% FBS, and 2% penicillin-streptomycin, and allowed to grow 48 h before analysis by immunofluorescence (see below).

Immunofluorescence Analysis and Apical Cell Surface Biotinylation of Cultured Epithelial Cells

Rab3B was immunolocalized in cultured epithelial cells using the protocol of Chavrier et al. (5). Cells grown on coverslips or filters were rinsed briefly in PBS (140 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCL, and 1.5 mM KH_2PO_4) and then prepermeabilized in 0.5% saponin in the following buffer (80 mM K-Pipes [pH 6.8], 5 mM EGTA, and 1 mM $MgCl_2$) for 5 min. The cells were then fixed in 3% formaldehyde in PBS for 15 min and washed in 0.5% saponin/PBS for 5 min. Free aldehydes were quenched by incubating the cells in 50 mM NH_4Cl in PBS for 10 min. The cells were washed again in 0.5% saponin/PBS for 5 min and incubated with the appropriate IgG at the indicated concentration (see Results) in 0.5% saponin/PBS for either 30 min at 37°C or overnight at 4°C. The cells were then washed three times in 0.5% saponin/PBS for 15 min and exposed to the secondary antibody (TRITC swine anti-rabbit IgG [Dako Corp., Carpinteria, CA]) at a 1:50 dilution in 0.5% saponin/PBS for 30 min at 37°C. The cells were washed again in 0.5% saponin/PBS and then extensively washed in detergent-free PBS. COS-7 cells were stained using the same protocol, with the omission of the saponin prepermeabilization step. For those double-label experiments in which the distributions of rab3B and desmoglein were compared, the rab3B staining was performed first followed by incubation with a mouse monoclonal antibody against desmoglein (Boehringer Mannheim Corp., Indianapolis, IN; 1:75 dilution) and then FITC anti-mouse IgG (Dako Corp.; 1:50 dilution), each for 1 h at 37°C.

Apical cell surface proteins were biotinylated as follows. First, T_{84} cells grown on filters were washed three times in ice-cold PBS and then exposed on the apical side to 5 μg/ml NHS-SS-biotin (Pierce Chem. Co.) in PBS at 4°C for 30 min. The filters were washed three times in cold PBS and then stained for rab3B immunofluorescence (see above) and with Hoechst nuclear stain (4 μg/ml) in PBS for 10 min at 4°C. Biotinylated proteins were subsequently stained with 5 μg/ml FITC-avidin (Cappel, Organon Teknika Corp., Durham, NC) for 10 min at 4°C.

Immunofluorescence images were collected using a slow scan, cooled CCD camera (Photometrics Inc., Woburn, MA) interfaced to an inverted microscope (Zeiss IM-35; Carl Zeiss, Inc., Thornwood, NY). Focal position was monitored using a Z-axis probe (Heidenhain) attached to the nose piece of the microscope.

Immunofluorescence Analysis of Semi-thin Frozen Sections of Mouse and Rat Tissue

Unfixed but fresh wedges of kidney, liver, and intestine were obtained from anesthetized mice and rats and snap frozen in isopentane chilled in a dry ice–acetone bath. Frozen sections were air dried, labeled with anti-rab3B, anti-Z0-1 (mAb kindly provided by B. Stevenson, University of Alberta, Edmonton, Alberta), and appropriate secondary antibody conjugates, and examined. Immunofluorescence images were collected as described above.

Immunoelectron Microscopy

Filter-grown T_{84} cells were rinsed in PBS and then permeabilized for 1 min in 0.1% Triton X-100 in PBS containing Ca^{2+} (0.1 mM) and Mg^{2+} (1 mM). Monolayers were then rinsed in PBS and fixed in 3% formaldehyde in PBS for 45 min. After extensive washing in PBS, nonspecific binding of IgG was blocked by incubation with 50% normal goat serum in PBS for 10 min at room temperature. Monolayers were then exposed to anti-rab3B IgG (100 μg/ml) or an equivalent concentration of preimmune IgG in 1% normal goat serum plus 0.1% BSA in PBS for 1 h at 37°C. After washing in PBS, the monolayers were then incubated with goat anti-rabbit IgG conjugated to 5-nm gold beads in 1% goat serum plus 0.1% BSA in PBS for 2 h at room temperature. After rinsing the monolayers in PBS followed by 0.1 M cacodylate buffer, they were postfixed first in 2.5% glutaraldehyde in cacodylate buffer for 1.5 h followed by 1% osmium tetroxide in cacodylate buffer for 1 h. Monolayers were then dehydrated in a graded ethanol series, embedded in Polybed resin, and thin sectioned for electron microscopic analysis.

Results

Rab3B mRNA Expression in Cultured Epithelial Cell Lines

Fig. 1 shows that cultured epithelial cells express rab3B mRNA at levels sufficient for detection by RT-PCR and by Northern analysis. The complete coding regions for rab3B and the broadly expressed rab5 were amplified by RT-PCR from mRNA that was isolated from cultured epithelial cells (i.e., human pancreatic cells [CFPAC [31]; Fig. 1] and human colonic cells [T_{84} [7]; not shown]). Each of these RT-PCR products was confirmed to represent the authentic coding region for rab3B or rab5 by DNA sequencing. The human rab3B clone that was generated from epithelial mRNA by RT-PCR was then used as a cDNA probe for Northern analysis. This probe recognized a single mRNA species in T_{84} and CFPAC epithelial cells, but not in rat PC12 neuroendocrine (12) or mouse fibroblasts (L cells). These results imply that rab3B is preferentially expressed in epithe-

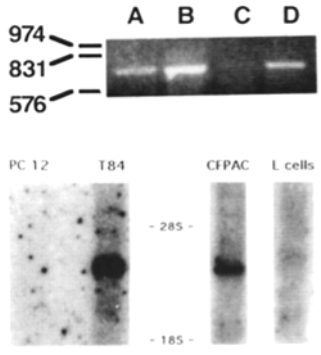


Figure 1. Rab3B mRNA expression in epithelial cell lines. (*Top*) RT-PCR amplification of the coding regions for rabs 3B and 5. (*A*) Rab3B amplified from CFPAC (epithelial) mRNA. (*B*) Rab5 amplified from CFPAC RNA. (*C*) PC12 mRNA amplified with rab3B primers. (*D*) Rab5 amplified from PC12 RNA. Locations of DNA markers indicated at left margin. (*Bottom*) Northern blots performed on poly(A)$^+$ RNA isolated from the following cell types: PC12 neuroendocrine cells, T_{84} epithelial cells, CFPAC epithelial cells, and mouse fibroblasts (*L cells*). Each lane 10 μg RNA. Ribosomal RNA bands (*28S* and *18S*) were visualized by staining each agarose gel with ethidium bromide prior to transferring the RNA to nylon membrane. Ribosomal RNA bands were of comparable intensity for all lanes (data not shown).

lial cells, as confirmed by immunoblotting and by immunofluorescence (see below).

Generation and Characterization of Rab3B Antibodies

We generated a rab3B-specific antibody for the purpose of confirming that rab3B protein is expressed in epithelial tissues, and to examine the intracellular targeting of this monomeric GTPase. Toward this end we expressed recombinant rab3B protein in bacteria as a fusion with GST and raised antisera against purified fusion protein. Antibody specificity was optimized by affinity purifying the antisera against a COOH-terminal polypeptide fragment of rab3B (CRab3B; see Materials and Methods). This region of rab3B (amino acids 168–219) exhibits little homology with other members of the rab protein family (~40% homology with rab3 isoforms, less than 20% amino acid identity with all other rabs; [2, 6, 19, 39]). Fig. 2 shows that the affinity-purified rab3B antibody was highly specific for rab3B versus rab3A and rab5 in immunoblots performed on recombinant fusion proteins.

The rab3B antibody was characterized further by expressing recombinant rab proteins in COS-7 cells and detecting the expressed proteins by immunofluorescence (Fig. 3). The rab3B antibody recognized recombinant rab3B, but not rabs 3A, 5, or 8, when each of these proteins was overexpressed in COS-7 cells. To our surprise, even the rab3B IgG that was not affinity purified against the hypervariable C-terminal domain (i.e., IgG isolated from rab3B antiserum) exhibited negligible cross reactivity with rab3A that was markedly

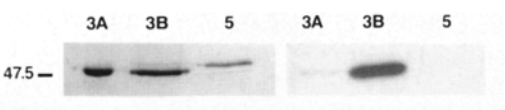


Figure 2. Immunoblot of recombinant fusion proteins performed using affinity purified rab3B antibody. (*Left*) 5-μg protein samples resolved by SDS-PAGE and stained with Coomassie blue. (*Right*) 10-ng protein samples blotted with affinity-purified rab3B antibody (10 μg/ml).

overexpressed in COS-7 cells. Rab3A-transfected COS cells were confirmed to overexpress this protein using a rab3A-specific antibody provided by T. Südhof (Fig. 3). COS-7 cells that were expressing rabs3A and 3B exhibited similar patterns of immunoreactivity; specifically, perinuclear staining and a necklace-like pattern of punctate staining in the cell periphery. This latter pattern of staining was not exhibited by other proteins that we have expressed in COS-7 cells (e.g., rab5 and CFTR; data not shown) and, therefore, may represent a relatively specific pattern of targeting for these two rab3 isoforms in COS-7 cells.

Expression and Targeting of rab3B Protein in Cultured Epithelial Cell Lines

Fig. 4 confirms that rab3B protein is detectably expressed in a number of epithelial cell lines. A single protein of the expected size was detected in immunoblots of light microsomes isolated from T_{84} and CFPAC epithelial cells, as shown in Fig. 4 *a*. Fig. 4 *b* summarizes the results of a survey of rab3B expression in a variety of cultured cell lines, where rab3B expression was assayed by immunoblotting of cell lysates. The highest levels of expression were detected for a differentiated clone of the HT29 human colonic epithelial cell line (HT29-CL19A; [1]). Conversely, no expression was detected in the parental HT29 cell line (which is undifferentiated [1]), or in a variety of nonepithelial cells including PC12 cells, bovine aortic endothelial cells, Hela cells (not shown), and mouse L cells (not shown). (Note that rab proteins are extremely well conserved from mouse to human [typically 97–100% amino acid identity], thus, these negative results are not due to species differences.) PC12 cells do express the brain-specific rab3 isoform, rab3A, as shown in Fig. 4 *b*, whereas no rab3A protein could be detected in any of the epithelial cell lines by immunoblotting (data not shown). These results support the notion that rab3B is preferentially expressed in certain epithelial cells. The lack of rab3B expression in undifferentiated HT29 cells, in contrast to the marked expression in differentiated colonic epithelial cells (i.e., HT29-CL19A and T_{84}), implies that the expression of this protein is developmentally regulated.

The results of the immunofluorescence experiments summarized in Figs. 5–7 indicate that rab3B preferentially targeted to regions of cell contact at the apical poles of cultured epithelial cells. Fig. 5 shows representative immunofluorescence micrographs of T_{84} cells grown on glass coverslips. Specific rab3B staining (i.e., staining not observed in the preimmune controls) was detectable as a ring-like pattern of fluorescence encircling each cell in the vicinity of the junctional complex, as well as occasional punctate fluorescence near the apical surface. Generally similar patterns of staining were observed for HT29-CL19A and CFPAC epithelial cells (e.g., see Fig. 11). Although we show only the results obtained using a single fixation protocol (see Materials and Methods), we observed similar patterns of staining using a variety of other protocols (e.g., methanol fixation). Conversely, we observed no specific rab3B staining of PC12 cells, Hela cells, or mouse L cells.

To document the polarity of rab3B targeting in cultured epithelial cells, we collected a series of optical sections of rab3B immunofluorescence staining of T_{84} cells cultured on permeable, transparent filters (Fig. 6). T_{84} cells cultured on

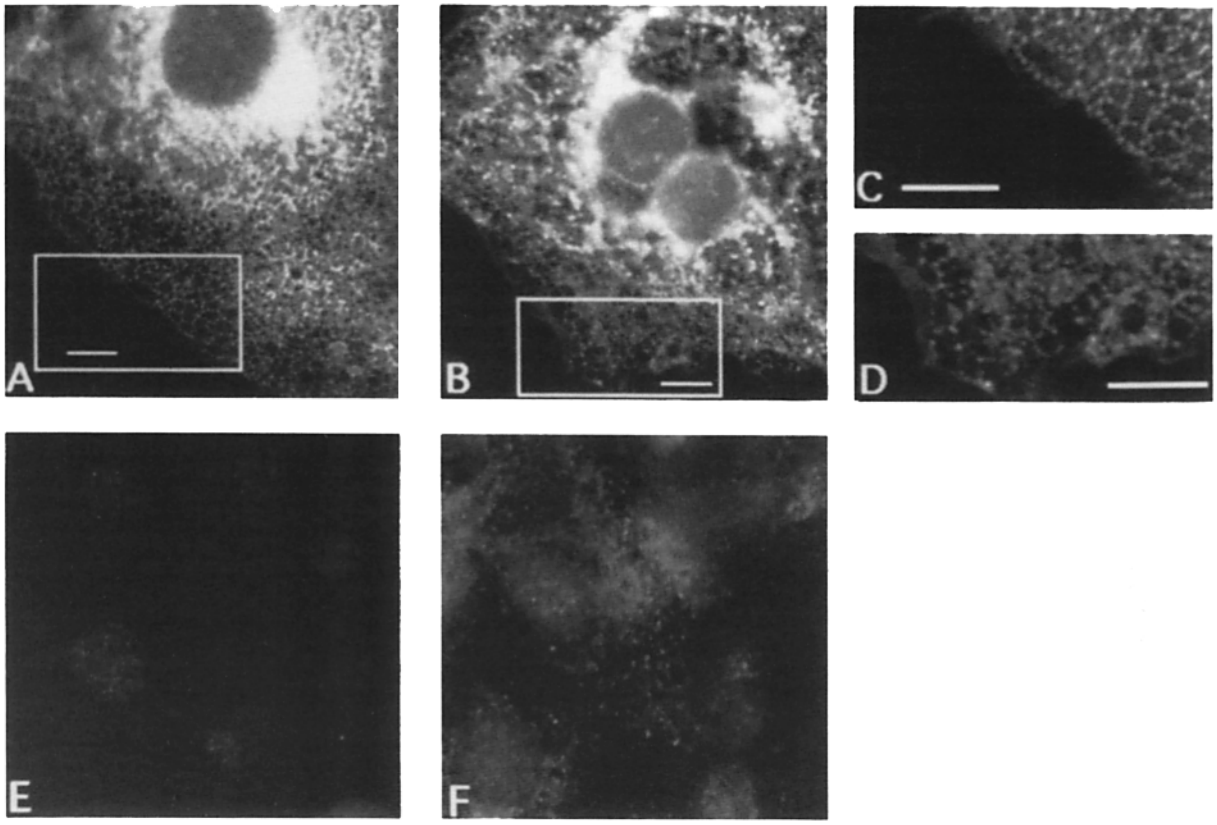


Figure 3. Immunofluorescence analysis of the expression of recombinant rabs3A and 3B in COS-7 cells. (*A* and *E*) COS-7 cells transfected with rab3B coding region and stained with anti-rab3B IgG (*A*) or rab3A-specific antibody (*E*). (*B* and *F*) COS-7 cells transfected with rab3A cDNA and stained with rab3A-specific antibody (*B*) or anti-rab3B IgG (*F*). (*C* and *D*) enlarged views corresponding to windows depicted in *A* and *B*, respectively. Bar, 5 μm.

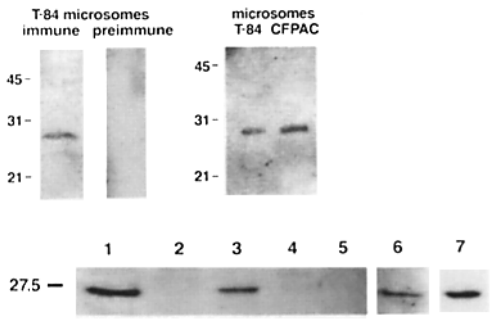


Figure 4. Rab3B protein expression in epithelial cell lines. (*Top*) Immunoblots of light microsomal membranes. (*Left*) Membranes prepared from T_{84} cells (40 μg protein per sample) and blotted with IgG fraction isolated from rab3B antiserum (14 μg/ml) or an equivalent concentration of preimmune IgG. (*Right*) membranes prepared from T_{84} and CFPAC cells (40 μg protein per sample) and blotted with affinity purified rab3B antibody (7 μg/ml). (*Bottom*) Immunoblots of cell lysates. (Lanes *1–5*) HT29-CL19A, HT29 (undifferentiated), T_{84}, PC12, bovine aortic endothelial cells (all blotted with 15 μg/ml rab3B IgG). (Lane *6*) HT29-CL19A cells blotted with affinity-purified rab3B antibody (10 μg/ml). (Lane *7*) PC12 cells blotted with rab3A-specific antibody. All protein samples: 50 μg.

this substrate form electrically resistive monolayers with relatively tall cells (~15 μm) and well developed tight junctions (7, 18). Fig. 6 shows the results of a triple-label experiment in which the apical membranes of filter-grown T_{84} cells were biotinylated and the cells were then stained with: (*a*) a fluorescent-avidin conjugate; (*b*) a nuclear stain (Hoechst); and (*c*) rab3B antibody. The rab3B staining was in the same focal plane as the biotinylated apical cell surface, where it was largely localized to regions of cell contact (although some en face staining was also detectable). Fig. 7 compares the distribution of rab3B to that of desmoglein, a desmosomal protein (30). This figure makes two points. First, the rab3B staining was localized to a very thin band of fluorescence at the apical cell surface, as evidenced by the fact that this staining was out of focus 1 μm below the apical surface. Second, the distribution of rab3B was distinct from that of desmoglein, which localized to the lateral cell membranes at regions basal to the predominant rab3B staining.

We have confirmed the targeting of rab3B near apical junctional complexes in cultured epithelial cells by immunoelectron microscopy, as shown in Fig. 8. Clusters of immunogold beads near tight junctions were routinely observed in T_{84} monolayers incubated with rab3B antibody. Conversely, no

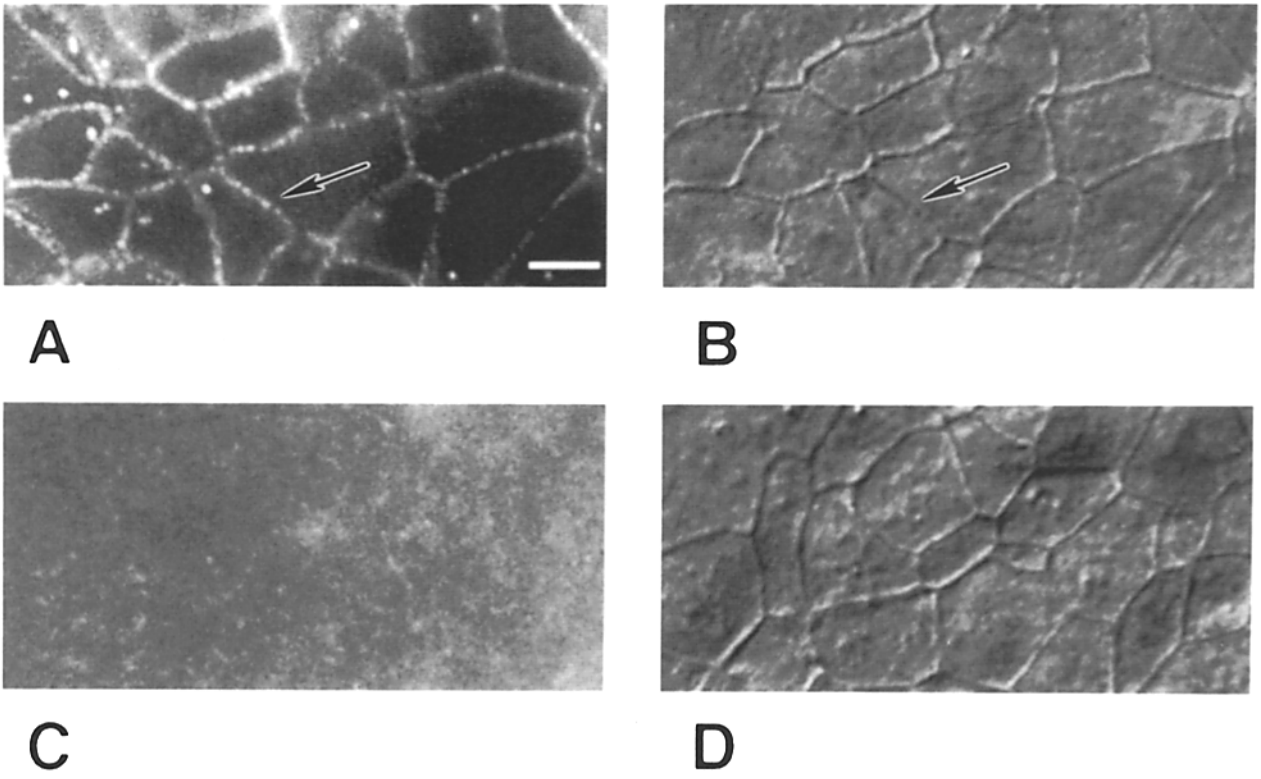


Figure 5. Rab3B immunofluorescence staining of T_{84} epithelial cells. (*A*) Immune staining obtained with anti-rab 3B IgG (50 μg/ml). (*B*) Corresponding Nomarski image. (*C*) Preimmune staining obtained with equivalent concentration of preimmune IgG. (*D*) Corresponding Nomarski image. Focal plane at apical cell surface for all images. Tight junctions appear as ribbons (see *arrow*) in Nomarski images. Bar, 5 μm.

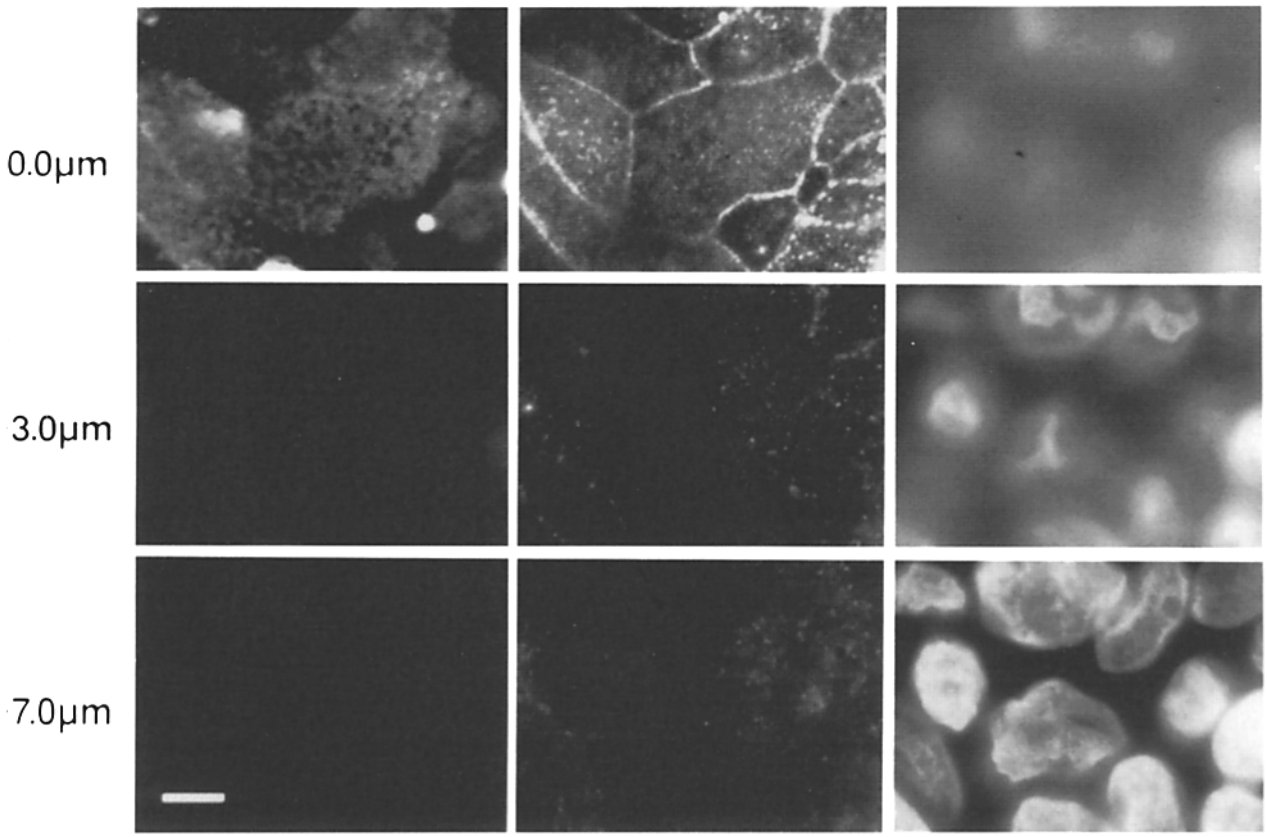


Figure 6. Polarized targeting of rab 3B in T_{84} epithelial cells cultured on permeable filters. Triple label experiment in which the apical cell surface was biotinylated and the cells were then labeled with FITC-avidin (*left*), anti-rab 3B IgG followed by TRITC anti-rabbit IgG (*middle*) and Hoechst nuclear stain. (*Right*) Optical sections were obtained at the apical cell surface (*0 μm*) and at 3 and 7 μm below the apical membrane.

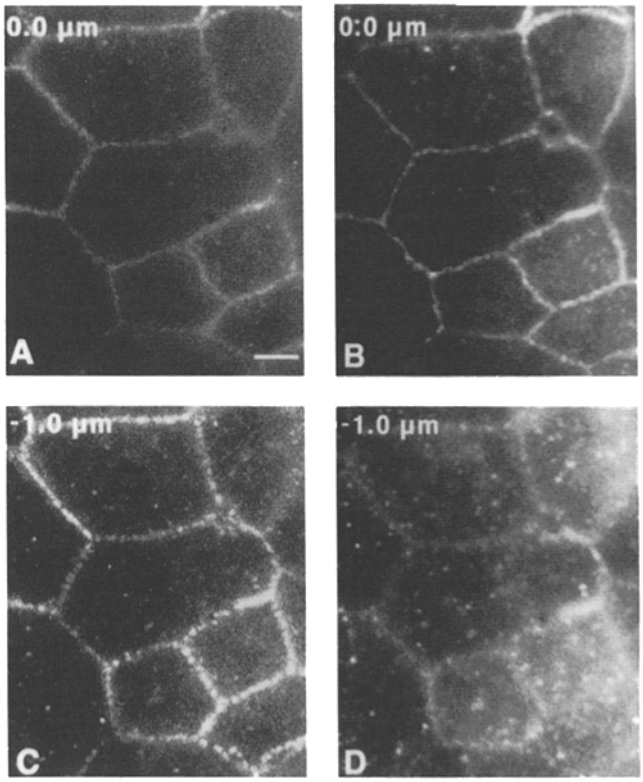


Figure 7. Optical sections of rab 3B and desmoglein immunofluorescence staining of T_{84} cells cultured on permeable filters. (*A* and *C*) Desmoglein immunofluorescence at apical cell surface (*0 μm*) and 1 μm below apical surface. (*B* and *D*) Rab 3B immunofluorescence at indicated focal positions. Bar, 5 μm.

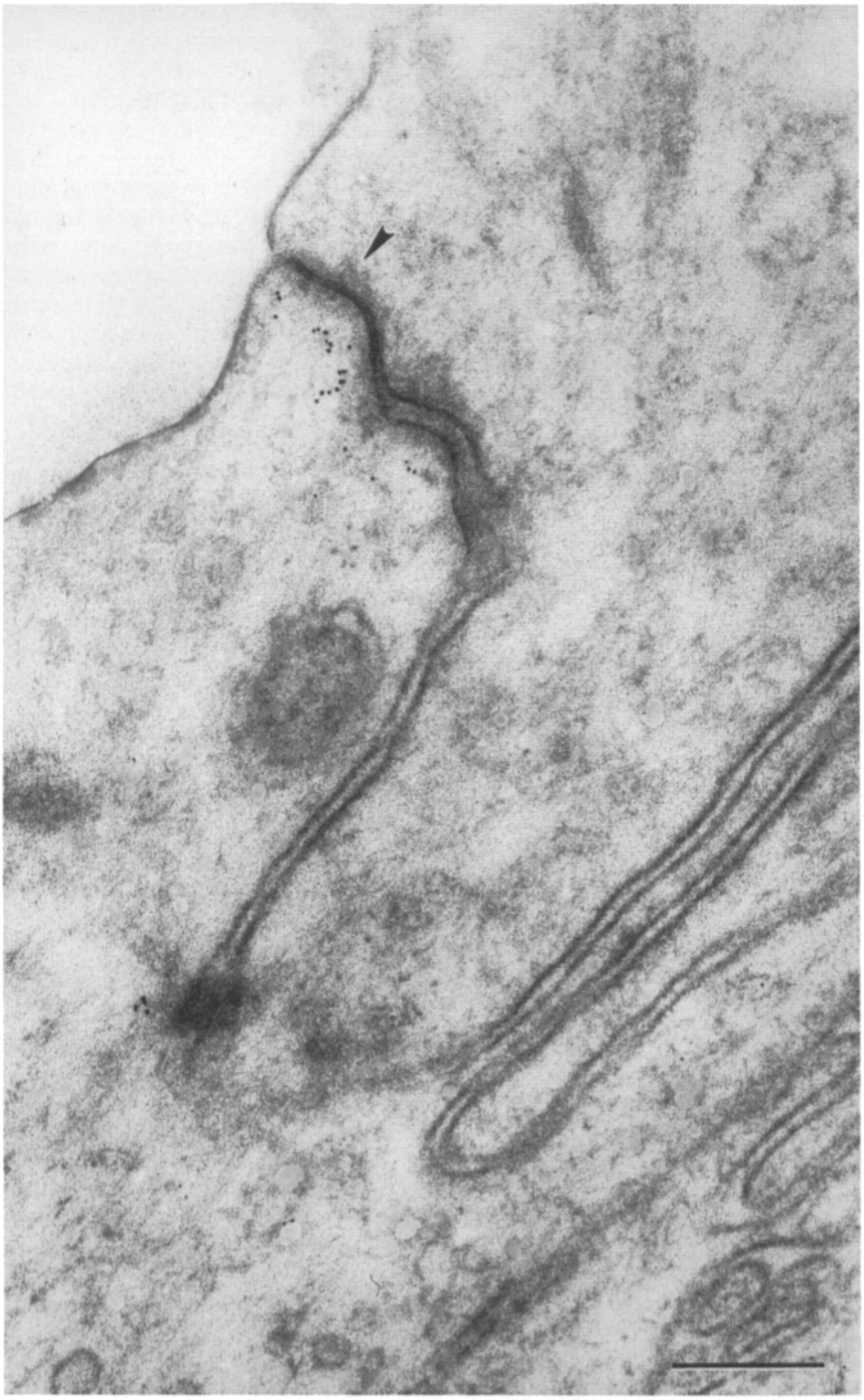
more than a single bead was ever observed near the tight junctions in the preimmune controls (out of greater than 500 junctions examined from 9 different control monolayers). The distance between the immunogold beads and the tight junctions was somewhat variable, ranging from 25 to 150 nm. We consider these distances to be rough estimates of the spacing between rab3B and the junctional complex, because of the dimensions of the antibody-gold complex (20–30 nm) and the membrane extraction that presumably accompanied the permeabilization of these cells by detergent (without which we could obtain no reliable immunogold labeling). These technical considerations prevent us from making more definitive statements about the ultrastructure of the membrane compartment with which rab3B associates.

Rab3B Expression and Targeting in Native Tissues

Rab3B expression in tissues isolated from mouse and rat was surveyed by immunofluorescence for two reasons: (*a*) to verify that rab3B is expressed in native epithelial tissues and exhibits polarized targeting within these tissues; and (*b*) to provide additional insights into the tissue distribution of this protein. No specific immunoreactivity was detected in the following tissues: brain, heart, skeletal muscle, smooth muscle, and endothelium (not shown). Conversely, we observed specific rab3B immunoreactivity in all mouse and rat epithelial tissues examined, with the exception of the renal glomerulus and proximal nephron (see Figs. 9 and 10). Positive epithelia included both crypt and villus of the small intestine, colon, hepatocytes of liver, and distal nephron. Within the colon, rab3B immunoreactivity was detectable in the surface epithelial cells (i.e., goblet cells and enterocytes), as well as in the epithelial cells lining the crypts. No differences in staining along the crypt axis were noted, although the staining within the crypts was greater than that in the surface epithelial cells. As with cultured epithelial cells, rab3B immunoreactivity within native mouse and rat tissues was typically localized to the apical poles near regions of cell contact. The polarity of rab3B targeting was most clearly evident in cross sections of distal nephron and colonic crypt, whereas the proximity of rab3B to regions of cell contact was obvious in en face images of hepatocytes, enterocytes, and renal epithelial cells. The targeting of rab3B near junctional complexes in native epithelia is most convincingly demonstrated by the results of the double-label immunofluorescence experiment shown in Fig. 9, *D* and *E*, in which sections of mouse colon were labeled with the rab3B polyclonal antibody and a monoclonal antibody raised against the tight junction protein, ZO-1 ([36]; antibody provided by B. Stevenson, University of Alberta, Edmonton, Alberta). The results of our analysis of rab3B expression in native tissues support the following conclusions. First, this monomeric GTPase is expressed in a variety of epithelial cells with major functions as diverse as protein secretion (i.e., hepatocytes and intestinal goblet cells), fluid secretion (i.e., colonic crypt cells), and fluid reabsorption (i.e., intestinal enterocytes and renal epithelial cells). Second, rab3B targets in a polarized fashion within all epithelial cells in which it is expressed, namely, to the apical pole near junctional complexes.

Regulation of rab3B Targeting by Cell Contact

The biogenesis of the epithelial tight junction and the concomitant development of cell polarity are processes that are regulated by cell contact (10, 37). We were interested in determining if rab3B targeting within epithelial cells is also regulated by cell contact, since its intracellular distribution implicates this monomeric GTPase as a candidate regulator of pathways of polarized membrane traffic (i.e., pathways that could participate in junction formation and/or cell polarization). The standard protocol for manipulating epithelial cell contact is the Ca^{2+} switch protocol, in which epithelial cells are first dissociated by incubation in Ca^{2+}-free media (i.e., to break down desmosomes and junctional complexes), and then switched back to Ca^{2+}-containing media to initiate cell contact and junction reassembly. Fig. 11 shows the results of such an experiment performed on HT29-CL19A cells. Cells that were dissociated by reducing the extracellular Ca^{2+} concentration either by incubation in nominally Ca^{2+}-free media or by the addition of EGTA exhibited a reversible retrieval of rab3B from the cell periphery. Several distinct stages in this process were apparent, although the time course varied depending on cell density (i.e., completely confluent monolayers dissociated more slowly). The first stage involved an unzippering of the rab3B immunofluorescence staining at the lateral cell margins into two distinct tracts, which represented the separate contributions of the opposing cells to the rab3B staining (Fig. 11, *B* and *C*). The second distinct stage involved a condensation of the rab3B staining into a circular profile that migrated to the extreme apical pole as each cell rounded up following junction disas-



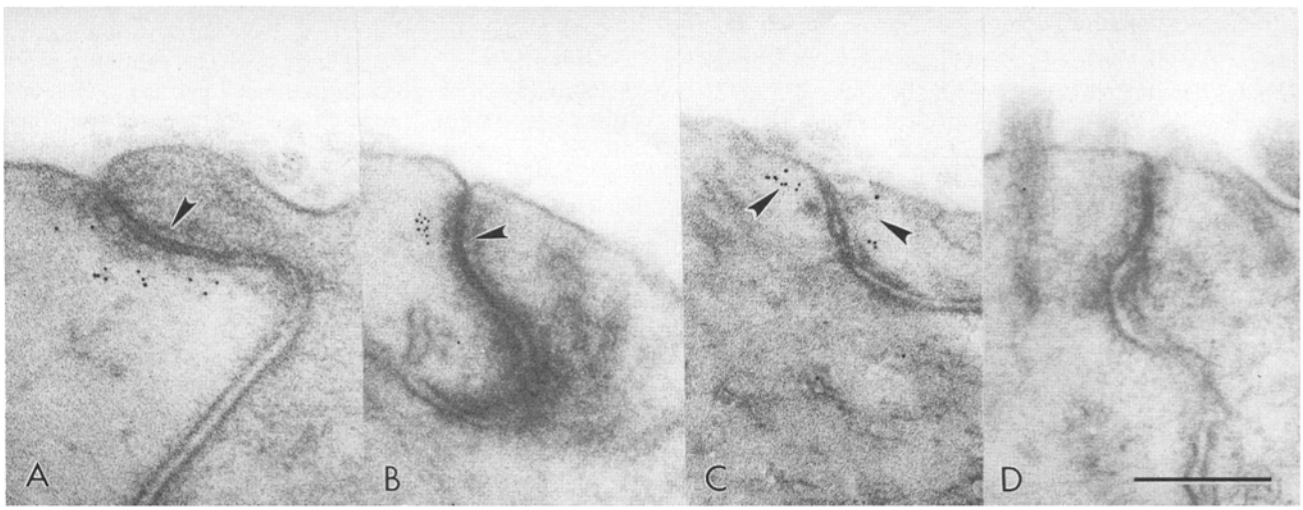


Figure 8. Immunoelectron microscopic localization of rab3B in T_{84} epithelial cells grown on permeable filters. (*Top*) Wide field view showing localization of immunogold beads near apical junctional complex (*arrow*). (*Bottom*) Representative patterns of immunogold labeling near junctional complexes of monolayers labeled with rab3B antibody (*A–C*) or with equivalent concentration of preimmune IgG (*D*). Cell-to-cell variability in labeling presumably reflects variations in cell permeabilization. Bar, 0.25 μm.

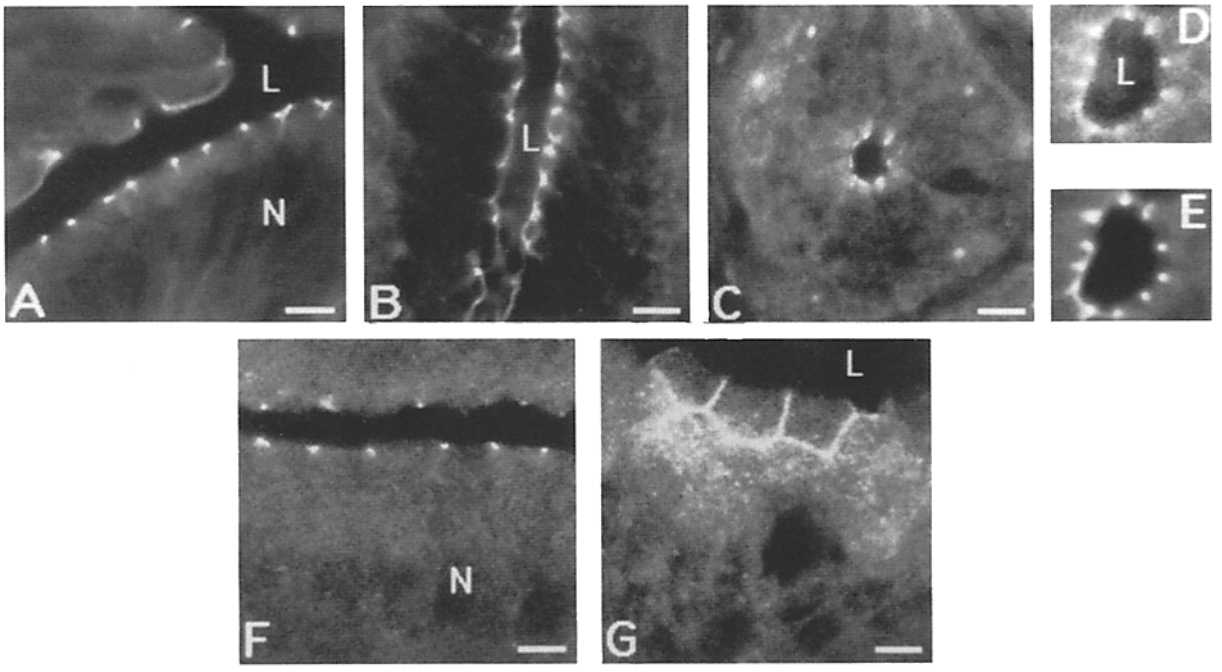


Figure 9. Rab3B expression in intestinal epithelia from mouse. (*A*) Apical staining of surface epithelium from large intestine (*L*, lumen; *N*, nucleus). Punctate staining represents staining at junctional complexes, as determined in double-label experiments performed using rab3B polyclonal antibody and ZO-1 monoclonal antibody (e.g., *D* and *E*). (*B*) Longitudinal section of colonic crypt with predominant rab3B staining at apical surface (*L*, lumen). (*C*) Cross section of colonic crypt stained with affinity-purified rab3B antibody. (*D* and *E*) Enlarged view of apical surface of colonic crypt double-labeled with rab3B IgG (*D*) and ZO-1 monoclonal antibody (*E*). (*F*) Surface epithelium from small intestine with punctate rab3B staining at junctional complexes (*N*, nucleus). (*G*) En face view of surface epithelial cells from small intestine showing predominant staining at junctional complexes (*L*, lumen). Equivalent staining patterns were observed with rab3B IgG (images *A*, *B*, *D–G*) and with affinity-purified rab3B antibody (e.g., *C*). No staining was observed with preimmune IgG. Bar, 5 μm.

sembly (Fig. 11 *D*). In the continued absence of extracellular Ca^{2+} (e.g., following overnight incubation without Ca^{2+}), this stage was then followed by a nearly complete loss of staining near the cell surface. Under this condition some cells (<5%) showed punctate rab3B staining at the few remaining points of contact between cells (Fig. 11, *E* and *F*). Many cells exhibited intracellular staining, with the most obvious pattern being a perinuclear localization (Fig. 11 *F*).

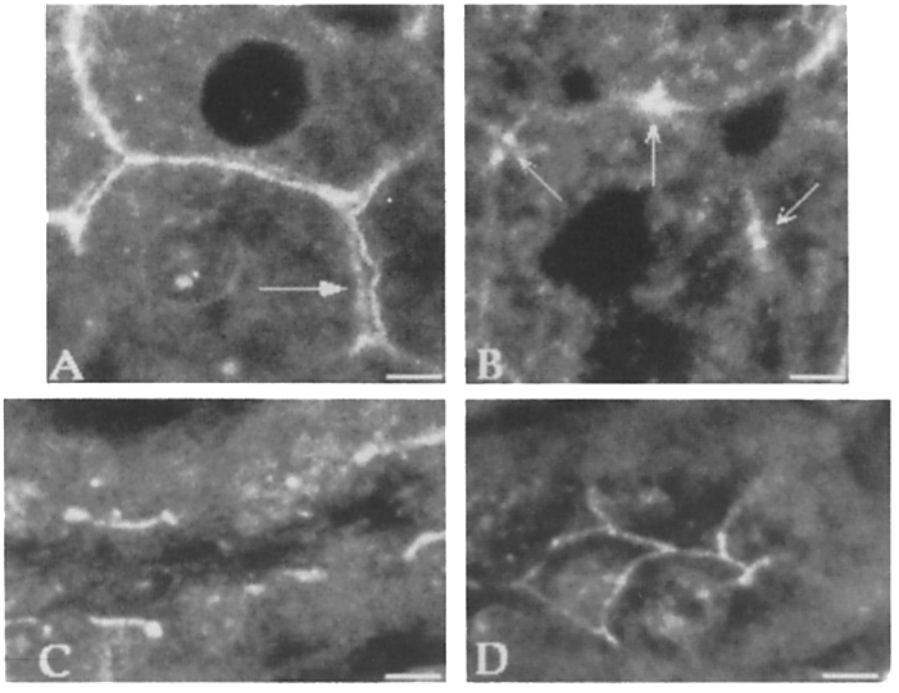


Figure 10. Rab3B expression in liver and kidney. (*A*) Section of mouse liver with rab3B staining along the canaliculi between adjacent hepatocytes (see *arrow*). (*B*) Section of mouse liver in which the canaliculi are transected sagittally, rather than longitudinally. Within this plane of section the rab3B immunofluorescence appears as discrete spots at the junctional complexes between adjacent hepatocytes (see *arrows*). Similar staining was obtained with the ZO-1 antibody (not shown). No staining along the sinuses (i.e., the hepatocyte basolateral membranes) was observed. (*C*) Longitudinal section of renal collecting duct from rat kidney. Note apical staining with rab3B antibody. (*D*) En face view of collecting duct cells with predominant staining at junctional complexes. Results obtained with rab3B IgG and with affinity-purified rab3B antibody (e.g., *B*) were identical. No staining with preimmune IgG was observed. Bar, 5 μm.

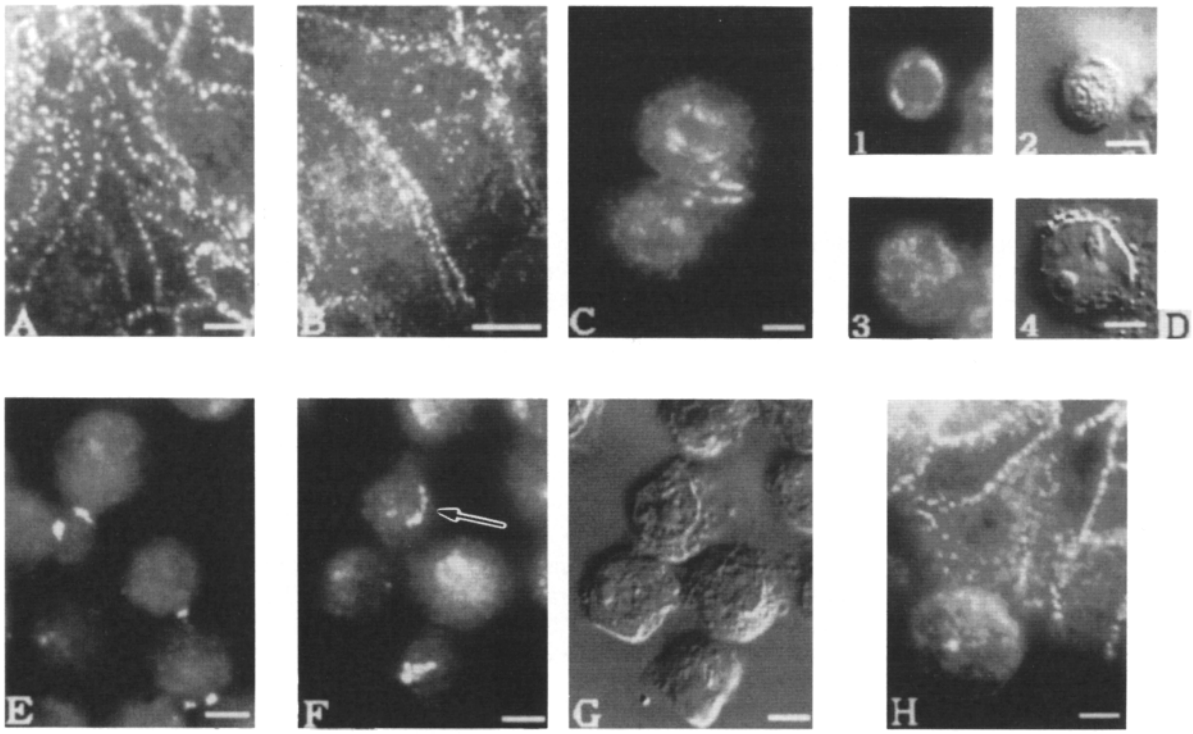


Figure 11. Regulation of rab3B targeting by cell contact. (*A*) Rab3B immunofluorescence staining of HT29-CL19a cells grown on glass coverslips in tissue culture medium containing 1.8 mM Ca^{2+}. Focal plane at apical cell surface. (*B*) Unzippering of rab3B staining at region of cell contact in cells incubated in low Ca^{2+} (*LC*) medium (Ca^{2+}-free Hank's media plus 0.8 mM Mg^{2+}) for 40 min. Focal plane at apical surface. Image was magnified twofold relative to others in this panel to improve resolution of the two parallel tracks of rab3B staining. (*C*) More complete breakdown of junctional rab3B staining in cells incubated in LC medium for 40 min. (*D*) Rab3B staining at surface of rounded cell that had been completely dissociated from its neighbors by exposure to LC medium for 70 min. Optical sections were collected at cell surface (*D1* and *D2*) and 9 μm below the cell surface at level of nucleus (*D3* and *D4*). (*E*) Rab3B staining at points of contact between cells that were grown overnight in Ca^{2+}-free medium (DME plus 2.5 mM EGTA). (*F*) Intracellular rab3B staining in cells grown overnight in Ca^{2+}-free medium (DME plus 2.5 mM EGTA). *Arrow*, perinuclear staining. (*G*) Nomarski image corresponding to *F*. (*H*) Recovery of rab3B staining at apical surface in cells that were first grown in Ca^{2+}-free medium for 1 d (DME plus 2.5 mM EGTA) and then returned to normal growth media for 2.5 d. Cells did not begin to form junctions until ~2 d following the Ca^{2+} switch, at which time rab3B staining became apparent at regions of cell contact. Bar, 5 μm.

This retrieval of rab3B could be reversed by switching back to Ca^{2+}-containing media (Fig. 11 *H*), which resulted in a reappearance of rab3B at regions of cell contact as HT29-CL19A cells gradually formed tight junctions (which in this cell line occurs over the course of several days; unpublished observations). Thus, rab3B redistributes from intracellular compartments to the region of the developing junctional complex as epithelial cells establish polarity following the Ca^{2+} switch.

Discussion

Our results provide definitive evidence for the polarized targeting of a member of the rab family of membrane traffic proteins (i.e., rab3B) within epithelial cells. We observed that rab3B is expressed in a wide variety of cultured epithelial cells and native epithelial tissues. Like other rab3 isoforms (e.g., the brain-specific rab3A and adipocyte-specific rab3D; [2]), this monomeric GTPase is expressed in a relatively tissue-specific fashion (i.e., it appears to be preferentially expressed in epithelial cells). Within epithelial monolayers rab3B is targeted to the apical pole proximate to the junctional complexes between polarized epithelial cells. Moreover, this intracellular targeting of rab3B is regulated by cell contact, involving a redistribution from intracellular compartments to the apical pole as epithelial cells establish polarity following the Ca^{2+} switch. This latter result raises the intriguing possibility that rab3B regulates membrane traffic pathways that participate in the development of the polarized phenotype, as discussed below.

Rabs 3A and 3B exhibit strikingly similar patterns of intracellular targeting in their respective tissues. Rab3A targets to regions of cell contact in undifferentiated PC12 neuroendocrine cells, and to the tips of neurites in differentiated PC12 cells (20). In polarized neurons this protein targets to the tips of axons (i.e., the presynaptic nerve terminal), where it probably regulates neurotransmitter release (9, 20, 21, 24, 26). Rabs 3A and 3B targeting to the nerve axon and the epithelial apical membrane, respectively, is perhaps not surprising, since these two membrane domains are equivalent destinations for the targeting of other classes of proteins as well (e.g., those membrane proteins anchored by glycosyl-phosphatidylinositol; [15]). Unlike those proteins, however, rabs 3A and 3B target to highly specialized regions in the nerve axon (i.e., the presynaptic terminal) and the epithelial apical pole (i.e., the junctional complex), respectively. This obser-

vation raises the interesting possibility that the nerve terminal and the epithelial junctional complex share certain design features and/or functional properties. This notion is supported by two additional observations. First, we have observed that rabs 3A and 3B both target to the tips of neurites when coexpressed in differentiated PC12 neuroendocrine cells (Weber, E., and K. L. Kirk, unpublished observations), as expected if the epithelial junctional complex and the nerve terminal are equivalent intracellular destinations for the targeting of these rab3 isoforms. Second, it has been reported recently that the tight junction associated protein, ZO-1 (36), exhibits considerable homology to a synaptic protein from rat brain (i.e., PSD-95; [38]). These observations imply that epithelial junctional complexes and nerve synapses have similar molecular architectures, which may reflect a common mechanism by which these specialized regions of cell contact develop and organize during the establishment of epithelial and neuronal polarity.

Rab proteins target to specific membrane compartments where they regulate distinct membrane traffic events. The targeting of rab3B near apical junctional complexes implies that this monomeric GTPase regulates the traffic of apical and/or junctional proteins. By analogy to rab3A, this protein conceivably regulates an apical exocytic pathway that is spatially segregated near regions of cell contact (i.e., near junctional complexes). That rab3B could regulate exocytosis is supported by recent evidence that antisense rab3B oligodeoxynucleotides inhibit exocytosis by anterior pituitary cells, which appear to express modest levels of rab3B (14). Moreover, the existence of an apical exocytic pathway that is spatially segregated near epithelial junctional complexes is suggested by evidence that an apical membrane protein (i.e., aminopeptidase) preferentially inserts into the apical plasma membrane at regions of cell contact in cultured renal epithelial cells (MDCK; [16]). Presumably, the protein cargo of such a spatially segregated pathway could vary among epithelial cell types (e.g., secreted proteins or ion channels to be inserted into the apical membrane), just as neurons vary in the cargo (i.e., neurotransmitters) that they secrete. Although this analogy to rab3A in neurons has its appeal, there may be differences between rabs 3A and 3B regarding the details of their participation in regulating such exocytic pathways. Specifically, whereas rab3A appears to associate with synaptic vesicles that are docked at the presynaptic nerve terminal, rab3B localizes to a region within polarized epithelial cells (i.e., near junctional complexes) that typically contains few secretory vesicles. Thus, in spite of the fact that these isoforms appear to target to the same membrane domains when coexpressed in the same cells (see above), they conceivably associate with qualitatively distinct membrane compartments within their respective native tissues.

The regulation of rab3B targeting by cell contact suggests another plausible function of rab3B; namely, the regulation of protein targeting to the junctional complex and apical membrane domain during epithelial polarization. Junctional proteins (e.g., ZO-1) and apical proteins are targeted from intracellular pools to the cell surface as epithelial cells establish polarity (10, 37). Interestingly, apical proteins are preferentially inserted at regions of cell contact during the polarization of MDCK cells (37) which implies the existence of a specific targeting patch at this region of the cell surface (23) that serves to direct the traffic of apical proteins to the plasma membrane. Such protein trafficking to regions of cell contact is a regulated process (e.g., by extracellular Ca^{2+}), and may be subject to the control of specific membrane traffic proteins; e.g., monomeric GTPases. The redistribution of rab3B from intracellular compartments to regions of cell contact places this monomeric GTPase in a position to regulate the recruitment of junctional proteins and/or apical proteins to the cell surface during epithelial polarization. This putative function of rab3B would be consistent with its apparently broad distribution among epithelial cells, since the generation of cell polarity is a common feature of epithelial tissues. We also note that such a role in epithelial cell development is not necessarily exclusive of this protein regulating apical exocytosis in fully differentiated epithelial cells. For example, two synaptic proteins in neuroendocrine cells (SNAP25 and synaptotagmin), each of which has been proposed to regulate neurotransmitter release, also may participate in the development of neuronal polarity (8, 25). Thus, rab3B, as a candidate regulator of polarized membrane traffic, could serve several important functions in epithelial tissues including the regulation of protein targeting pathways that underlie the development of epithelial polarity.

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